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Positive Regulation of Adult Bone Formation by Osteoblast-Specific Transcription Factor Osterix

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ABSTRACT: Osterix (Osx) is essential for osteoblast differentiation and bone formation, because mice lacking Osx die within 1 h of birth with a complete absence of intramembranous and endochondral bone formation. Perinatal lethality caused by the disruption of the *Osx* gene prevents studies of the role of Osx in bones that are growing or already formed. Here, the function of *Osx* was examined in adult bones using the time- and site-specific Cre/loxP system. *Osx* was inactivated in all osteoblasts by Col1a1-Cre with the activity of Cre recombinase under the control of the 2.3-kb collagen promoter. Even though no bone defects were observed in newborn mice, *Osx* inactivation with 2.3-kb *Col1a1-Cre* exhibited osteopenia phenotypes in growing mice. BMD and bone-forming rate were decreased in lumbar vertebra, and the cortical bone of the long bones was thinner and more porous with reduced bone length. The trabecular bones were increased, but they were immature or premature. The expression of early marker genes for osteoblast differentiation such as Runx2, osteopontin, and alkaline phosphatase was markedly increased, but the late marker gene, osteocalcin, was decreased. However, no functional defects were found in osteoclasts. In summary, *Osx* inactivation in growing bones delayed osteoblast maturation, causing an accumulation of immature osteoblasts and reducing osteoblast function for bone formation, without apparent defects in bone resorption. These findings suggest a significant role of Osx in positively regulating osteoblast differentiation and bone formation in adult bone.
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Key words: Osterix, Col1a1-Cre, osteopenia, osteoblast differentiation, adult bone formation

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INTRODUCTION

Bone is a dynamic, living tissue. The skeleton and its various skeletal elements are composed of two tissues, cartilage and bone, and three cell types, chondrocytes, osteoblasts, and osteoclasts.[1–3] The complexity of the bone regulation system involves the coupling between bone-forming osteoblasts and bone-resorbing osteoclasts because osteoblasts control the degree of osteoclastic activity.[4–6] Osteoblasts have been widely studied over the past century to improve our understanding of bone remodeling. These cells arise from osteoprogenitor cells in the periosteum and bone marrow. Osteoprogenitor cells differentiate into osteoblasts and, in fact, the mature bone cells are responsible for mineralization of the osteoid matrix, which is composed mainly of type I collagen. Osteoblasts trapped in the bone matrix become osteocytes, and these major mechanosensory cells in bone cease to generate osteoid and mineralized matrix.

Runx2 and *Osterix* (*Osx*) are master genes for osteoblast differentiation and function. *Runx2* is a well-characterized transcriptional regulator that is expressed in prehypertrophic chondrocytes and osteoblasts and plays multiple roles during chondrogenesis and osteogenesis.[7–9] *Runx2*-deficient mice or C terminus–truncated *Runx2* mice show a complete lack of both intramembranous and endochondral ossification caused by the absence of osteoblast differentiation.[7,10–12] *Osx* is a novel zinc finger-containing transcription factor that is essential for the differentiation of pre-osteoblasts into functional osteoblasts.[13] *Osx* homozygous null mutant mice show normal cartilage development but a complete absence of bone formation. Whereas no *Osx* expression occurs in *Runx2*-deficient mice, normal expression of *Runx2* is observed in osteoblasts of *Osx*-null mutant mice. This indicates that the *Osx* gene is downstream of *Runx2* and is an essential transcription factor for

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osteoblast differentiation.[13] However, because *Osx* null mutants die immediately after birth, it has not been possible to address critical questions regarding the possible role of Osx in the physiology of adult bones.

Based on previous mouse genetic studies, we hypothesized that Osx might play a significant role in osteoblast function and bone formation in adult mice. To test this hypothesis, conditional *Osx* knockout mice were generated to inactivate the *Osx* gene in osteoblasts under the control of a 2.3-kb type I collagen promoter (*Col1a1*), which can drive Cre expression at high levels in osteoblasts and odontoblasts and at a specific time (after embryonic day 14.5).[14–18] In this study, *Osx* deficiency in osteoblasts under the control of the *Col1a1* promoter ($Osx^{flox/-}$;*Col1a1-Cre*) resulted in osteopenia in the vertebrae and a thinner, more porous cortical bone phenotype in long bones with an arrest of bone turnover. These skeletal phenotypes were caused by the inhibition of osteoblast maturation and the accumulation of immature osteoblasts in the bone of adult mice and not functionally unaltered osteoclasts. Therefore, these results showed that Osx played a significant role in regulating osteoblast differentiation and bone formation in growing bone and during early bone development. Our data provide novel insight into the role of this critical transcription factor in osteoblast function and in bone maintenance after birth.

MATERIALS AND METHODS

Generation of conditional Osx knockout mice with Col1a1-Cre

$Osx^{flox/-}$;*Col1a1-Cre* were generated by crossing homozygous *Osx* floxed mice ($Osx^{flox/flox}$)[19] and $Osx^{+/-}$;*Col1a1-Cre* mice, which were obtained by crossing two $Osx^{flox/+}$ mice and by mating *Osx* heterozygous mice ($Osx^{+/-}$)[13] with *Col1a1-Cre* transgenic mice (unpublished data), respectively. In these mice, one *Osx* allele contained two loxP sites surrounding exon 2 and the other *Osx* allele was inactive and replaced by *LacZ* expressed under the regulatory sequences that normally control the *Osx* gene; these mice also harbored the *Col1a1-Cre* transgene with Cre recombinase, which is active in osteoblasts under the control of the 2.3-kb *Col1a1* promoter. In mice harboring these three alleles, routine mouse genotyping was conducted using tail genomic DNA. The flox allele was amplified to generate a 390-bp fragment compared with 300 bp for the wildtype allele using the following primers: 5′-CTTGGGA ACACTGAAGCTGT-3′ and 5′-CTGTCTTCACCTCAA TTCTATT-3′. The other primers were specific for the targeted allele with *LacZ* gene (5′-GCATCGAGCTGGG TAATAAGGGTTGGCAAT-3′ and 5′-GACACCAGA CCAACTGGTAATGGTAGCGAC-3′), the *Cre* transgene (5′-ATCCG-AAAAGAAAACGTTGA-3′ and 5′-A TCCAGGTTACGGATATAGT-3′), and the deleted *Osx* exon 2 (Δex) allele (5′-CTTGGGAACACTGAAGCTGT -3′ and 5′-GCAC-ACCGGCCTTATTCC-3′) to amplify 860-, 700-, and 544-bp fragments, respectively. All procedures concerning animal experiments were conducted with the approval of Kyungpook National University.

Histological and histomorphometric analysis

Mice were fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Long bones of postnatal mice were decalcified, embedded in paraffin, sectioned at 6–8 μm, and stained with H&E and Alcian blue. Von Kossa staining was performed in undecalcified vertebrae that were embedded in destabilized methyl-methacrylate according to standard protocols.[20] In vivo cell proliferation was assessed by BrdU incorporation (Zymed) and apoptotic cells were visualized by TUNEL analysis, using the TACS 2 TdT-DAB In Situ Apoptosis Detection Kit (Trevigen). For the assessment of dynamic histomorphometric indices, mice were injected with calcein at a dose of 30 mg/kg body weight at 6 and 2 days before death. Static and dynamic histomorphometric analysis was conducted using TAS image analysis systems (TAS; Leitz, Wetzlar, Germany), Bioquant programs (Bio-Quant), and the OsteoMeasure histomorphometry system (OsteoMetrics).[21] Statistical differences were assessed by the *t*-test.

μCT analysis

For 3D morphological and histomorphometric analysis, the mouse femur was scanned using the eXplore Locus SP scanner (GE Healthcare) at 8-μm resolution. All morphometric parameters were determined using eXplore MicroView version 2.2 (GE Healthcare). The mineralized tissues were differentially segmented by a global thresholding procedure. In the femora, three preselected regions were analyzed: whole bone, the distal metaphysis extending proximally 1.75 mm from the proximal tip of the primary spongiosa, and a diaphyseal segment extending 0.25 mm proximally and distally from the midpoint between the femoral ends.

TRACP staining and osteoclast activity assays

TRACP staining of osteoclasts was performed on deparaffinized bone sections according to the manufacturer's instructions. After incubation in TRACP reagent, sections were washed in water and counterstained with methyl green. In vivo osteoclast activity was measured in urine samples collected from sex- and age-matched mice. The urinary excretion of deoxypyridinoline (DPD) cross-links was determined using Metra DPD EIA (Quidel) and QuantiChromC reatinine Assay Kits (Bioassay) according to the manufacturer's instructions.

Quantitative real-time PCR analysis

Total RNA was isolated from long bones at 4 wk of age using TRI REAGENT (Sigma-Aldrich). RNA was subjected to quantitative real-time PCR using 2× SYBR Green Master mix reagent (Applied Biosystems). The following primers for marker genes of osteoblast and osteoclast differentiation were used: Osx, 5′-GCAACTGGC TAGGTG-GTGGTC-3′ and 5′-GCAAAGTCAGATGG GTAAGTAGGC-3′; Runx2, 5′-AAATGC-CTCCGCTG TTATGAA-3′ and 5′-GCTCCGGCCCACAAATCT-3′; Bsp, 5′-ACCCCA-AGCACAGACTTTTGA-3′ and 5′-C TTTCTGCATCTCCAGCCTTCT-3′; Col1a1, 5′-CCTGA

GTCAGCAGATTGAGAACA-3′ and 5′-CCAGTACTC TCCGCTCTTCCA-3′; osteocalcin (OCN), 5′-GCGCTCT GTCTCTCTGACCT-3′ and 5′-ACCTTATTGCCCT-CC TGCTT-3′; alkaline phosphatase (ALP), 5′-AACCCAGA CACAAGCATTCC-3′ and 5′-GCCTTTGAGGTTTTTG GTCA-3′; osteopontin (OPN), 5′-TGCACCCAGATCCT A-TAGCC-3′ and 5′-CTCCATCGTCATCATCATCG-3′; RANKL, 5′-GCAGAAGGAAC-TGCAACACA-3′ and 5′-GATGGTGAGGTGTGCAAATG-3′; osteoprotegerin (OPG), 5′-AGCTGCTGAAGCTGTGGAA-3′ and 5′-GG TTCGAGTGGCCGAGAT-3′; TRACP, 5′-CGACCATT GTTAGCCACATACG-3′ and 5′-TCGTCCTGAAGAT ACTGCAGGT-T-3′; cathepsin K (CathK), 5′-ATATGTG GGCCAGGATGAAAGTT-3′ and 5′-TCGTT-CCCCAC AGGAATCTCT-3′; and MMP9, 5′-GCCCTGGAACTCA CACGACA-3′ and 5′-TTGGAAACTCACACGCCAGA AG-3′. Three independent measurements per sample were performed. The quantified individual RNA expression levels were normalized to GAPDH and depicted as relative RNA expression levels with the corresponding heterozygous mice (*Osx$^{flox/+}$;Col1a1-Cre*) set to 1.0.

In vitro osteoblast culture and differentiation

Primary osteoblasts were isolated from calvaria of neonatal mice. For differentiation, cells were plated into 24-well culture dishes at a density of 1×10^5 cells/well and differentiated in vitro in medium supplemented with 5 mM β-glycerophosphate and 100 μg/ml ascorbic acid. After 3 wk of culture, bone nodules were identified morphologically by alizarin red and von Kossa staining.

RESULTS

Osteoblast-specific deletion of Osx with Col1a1-Cre rescues the perinatal lethality of Osx null mutant mice

To study the role of Osx in adult bones, mice harboring a conditional floxed allele of *Osx* (*Osx^{flox}*)[19] were used. By crossing the *Col1a1-Cre* transgenic mice with *Osx^{flox}* mice, the *Osx* gene was inactivated in osteoblasts after bone collar formation at mouse embryonic day 14.5. Finally, *Osx$^{flox/–}$;Col1a1-Cre* mice containing one conditional *Osx^{flox}* allele and one *Osx*-null allele,[13] as well as a *Col1a1-Cre* transgene, referred to as homozygote, were generated (Fig. 1A). *Osx$^{flox/+}$* and *Osx$^{flox/+}$;Col1a1-Cre* mice were also generated for wildtype and heterozygous controls, respectively (Fig. 1A). Mice were genotyped by PCR of tail genomic DNA to identify *Osx$^{flox/+}$*, *Osx$^{flox/+}$; Col1a1-Cre*, and *Osx$^{flox/–}$;Col1a1-Cre* (Fig. 1B). Unlike perinatal lethality by *Osx* gene disruption,[13] *Osx$^{flox/+}$; Col1a1-Cre* and *Osx$^{flox/–}$;Col1a1-Cre* mice were viable and analyzed as heterozygous control and null mutant, respectively.

Osteopenia in Osx$^{flox/–}$;Col1a1-Cre adult mice

Osx$^{flox/+}$;Col1a1-Cre and *Osx$^{flox/–}$;Col1a1-Cre* mice appeared phenotypically normal when they were born. To study the bone phenotype, skeletons of newborns were stained with Alcian blue for cartilage and alizarin red for calcified tissue (Fig. 1C). No skeletal abnormalities were observed in *Osx$^{flox/–}$;Col1a1-Cre* compared with *Osx$^{flox/+}$; Col1a1-Cre*. There was no significant difference in histological analysis with H&E, Alcian blue, and von Kossa staining (Figs. 1D–1F) between both newborn mice.

Because no clear differences were observed in newborn mice, the bones of *Osx*-inactivated mice with *Col1a1-Cre* were analyzed in adult mice at 8 and 16 wk of age when their bones reached peak bone mass.[22] Mineralized trabecular bones stained with von Kossa were remarkably reduced in *Osx$^{flox/–}$;Col1a1-Cre* at 8 wk of age (Fig. 2A). Although trabecular bones were slightly increased in *Osx$^{flox/–}$;Col1a1-Cre* at 16 wk compared with 8 wk of age, reduced trabecular bone volume was still observed in *Osx$^{flox/–}$;Col1a1-Cre* compared with *Osx$^{flox/+}$;Col1a1-Cre*. Histomorphometric analysis of the fourth lumbar vertebra showed a significant reduction in cancellous bone volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N), with an increase in trabecular separation (Tb.Sp) in *Osx$^{flox/–}$;Col1a1-Cre* (Fig. 2B). As a result, *Osx$^{flox/–}$;Col1a1-Cre* adult mice that lacked the *Osx* gene in osteoblasts failed to acquire as much bone as their control littermates and showed lower trabecular bone mass in the vertebrae throughout their lifetime.

Postnatal defect in osteoblast function in Osx$^{flox/–}$;Col1a1-Cre

To determine whether Osx had an effect on osteoblast function in *Osx$^{flox/–}$;Col1a1-Cre* mice, the bone forming rate (BFR) was examined by calcein double labeling, which permitted an assessment of the dynamic and static parameters of bone formation. Double labeling with fluorescence was clearly observed on the surfaces of lumbar bones of *Osx$^{flox/+}$;Col1a1-Cre* mice but not in *Osx$^{flox/–}$; Col1a1-Cre* mice (Fig. 2C). In the histomorphometric analysis, *Osx$^{flox/–}$;Col1a1-Cre* exhibited a 2-fold increase in the single labeling surface with a decrease in the double labeling surface (Fig. 2D). BFR, an indicator of osteoblast activity, was significantly reduced in *Osx$^{flox/–}$;Col1a1-Cre*, showing a functional defect of osteoblasts in vivo in *Osx$^{flox/–}$;Col1a1-Cre* adult mice. The mineral apposition rate (MAR), which indicates the rate of new bone deposition in the radial direction, was also remarkably decreased in *Osx$^{flox/–}$;Col1a1-Cre* (Fig. 2D). Consistent with the dominant nature of the osteopenic phenotype, these results supported the postulation that *Osx* inactivation in osteoblasts after bone collar formation reduced or delayed the function of osteoblasts in bone formation.

Alterations of cortical and trabecular bone with inhibition of long bone growth in Osx$^{flox/–}$;Col1a1-Cre

A more detailed characterization of the skeletal phenotype was obtained by μCT analysis. 3D μCT clearly showed a reduction of the femoral length in *Osx$^{flox/–}$; Col1a1-Cre*, reflecting an impaired growth of long bones in *Osx$^{flox/–}$;Col1a1-Cre* mice at 6 wk of age (Fig. 3A). A more porous osteopenic phenotype and reduced thickness of cortical bone was observed in diaphysis of *Osx$^{flox/–}$;Col1a1-Cre*

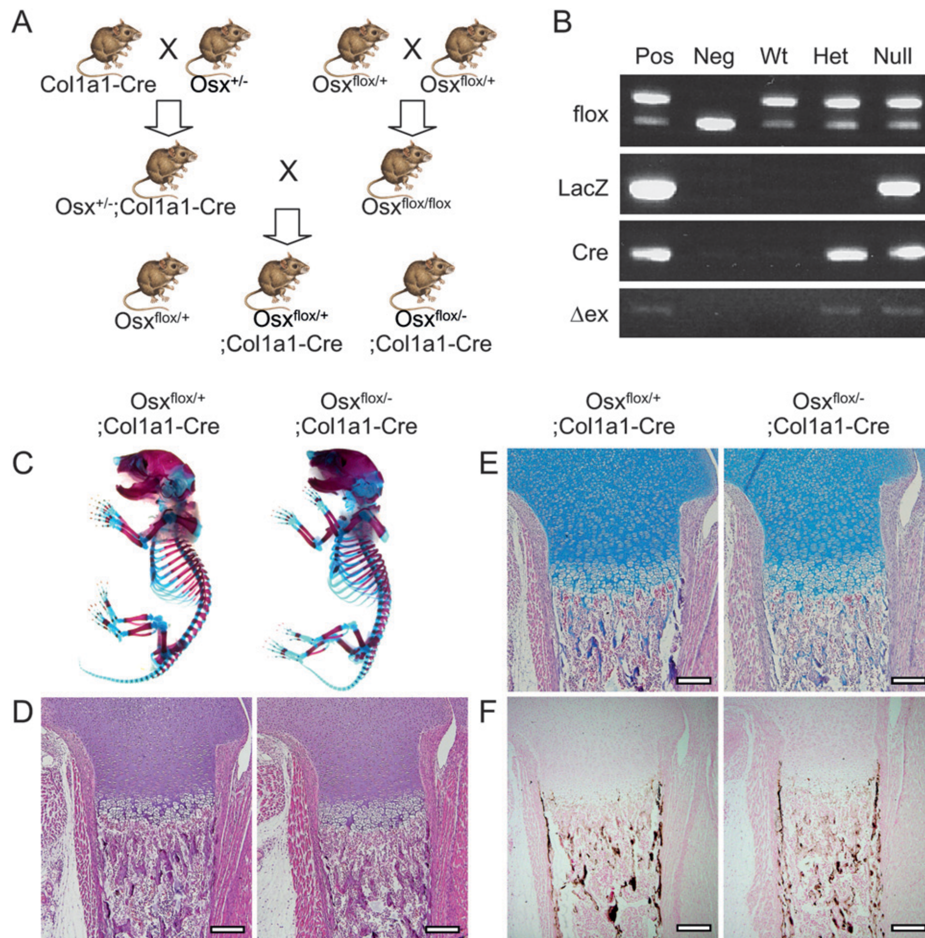

FIG. 1. Generation of *Osx$^{flox/–}$;Col1a1-Cre* mice. (A) Breeding scheme to generate *Osx$^{flox/+}$;Col1a1-Cre* and *Osx$^{flox/–}$;Col1a1-Cre* mice. *Col1a1-Cre* transgenic mice were used to excise *Osx* in osteoblasts. Finally, *Osx$^{flox/+}$*, *Osx$^{flox/+}$;Col1a1-Cre*, and *Osx$^{flox/–}$;Col1a1-Cre* were generated as wildtype, heterozygous, and null mutant mice, respectively. (B) PCR genotyping by tail genomic DNA. Tail genomic DNA was isolated in *Osx$^{flox/+}$* (Wt), *Osx$^{flox/+}$;Col1a1-Cre* (Het), and *Osx$^{flox/–}$;Col1a1-Cre* (Null) mice at P10. PCR was performed using four sets of primers, flox, LacZ, Cre, and Δex, for *Osx* floxed allele, *LacZ* knock-in allele, *Cre* transgene, and *Osx* exon 2 deletion, respectively. Pos, positive control; Neg, negative control. (C) Analysis of skeletal phenotypes in conditional *Osx* knockout mice with *Col1a1-Cre*. Skeletons of newborn mice were stained with Alcian blue for cartilage followed by alizarin red for bone. *Osx$^{flox/–}$;Col1a1-Cre* showed no bone defects compared with *Osx$^{flox/+}$;Col1a1-Cre*. (D–F) Histological analysis of femur was performed with H&E (D), Alcian blue (E), and von Kossa (F) staining. No difference between *Osx$^{flox/+}$;Col1a1-Cre* and *Osx$^{flox/–}$;Col1a1-Cre* newborn mice was observed in differentiating chondrocytes and mineralized bones by Alcian blue and von Kossa staining, respectively. Scale bar = 200 μm.

by μCT and peripheral QCT analysis, respectively (Fig. 3B). Compared with the histomorphometry of *Osx$^{flox/+}$;Col1a1-Cre* mice, the respective cortical area, BMD, and thickness were remarkably reduced in diaphysis of *Osx$^{flox/–}$;Col1a1-Cre* mice (Fig. 3C). In addition, the mean cortical outer and inner perimeters (Peri) were increased in *Osx$^{flox/–}$;Col1a1-Cre* (Fig. 3C), resulting in reduced cortical bone formation. The midsagittal sections of tibial bones showed an obvious abnormality of cortical bone (Fig. 3D). At 8 wk of age, thinner and more porous cortical bone was observed in *Osx$^{flox/–}$;Col1a1-Cre* compared with the normal concrete cortical bone in *Osx$^{flox/+}$;Col1a1-Cre*. Even though cortical bones were formed and thickened in *Osx$^{flox/–}$;Col1a1-Cre* at 16 wk compared with 8 wk of age, the cortical thickness of *Osx$^{flox/–}$;Col1a1-Cre* was still less than that of *Osx$^{flox/+}$;Col1a1-Cre* (Fig. 3D).

Trabecular bone morphology was confirmed in 2D longitudinal image by μCT analysis. With the osteopenic phenotype in cortical bone, many small pieces of trabecular bones were observed in *Osx$^{flox/–}$;Col1a1-Cre* (Fig. 4A). At the distal femoral metaphysis, trabecular bone volume (BV/TV) was higher in *Osx$^{flox/–}$;Col1a1-Cre* than *Osx$^{flox/+}$;Col1a1-Cre* at 6 wk of age (Figs. 4B and 4C). Whereas the trabecular number was increased, trabecular thickness and separation were significantly reduced in *Osx$^{flox/–}$;Col1a1-Cre*, indicating that trabecular bones in *Osx$^{flox/–}$;Col1a1-Cre* were immature like that observed in normal newborn or young mice or were premature because of unknown alterations in bone formation without *Osx* (Fig. 4C). However, the growth plate of *Osx$^{flox/–}$;Col1a1-Cre* appeared normal compared with that of *Osx$^{flox/+}$;Col1a1-Cre* mice, indicating that chondrogenesis was

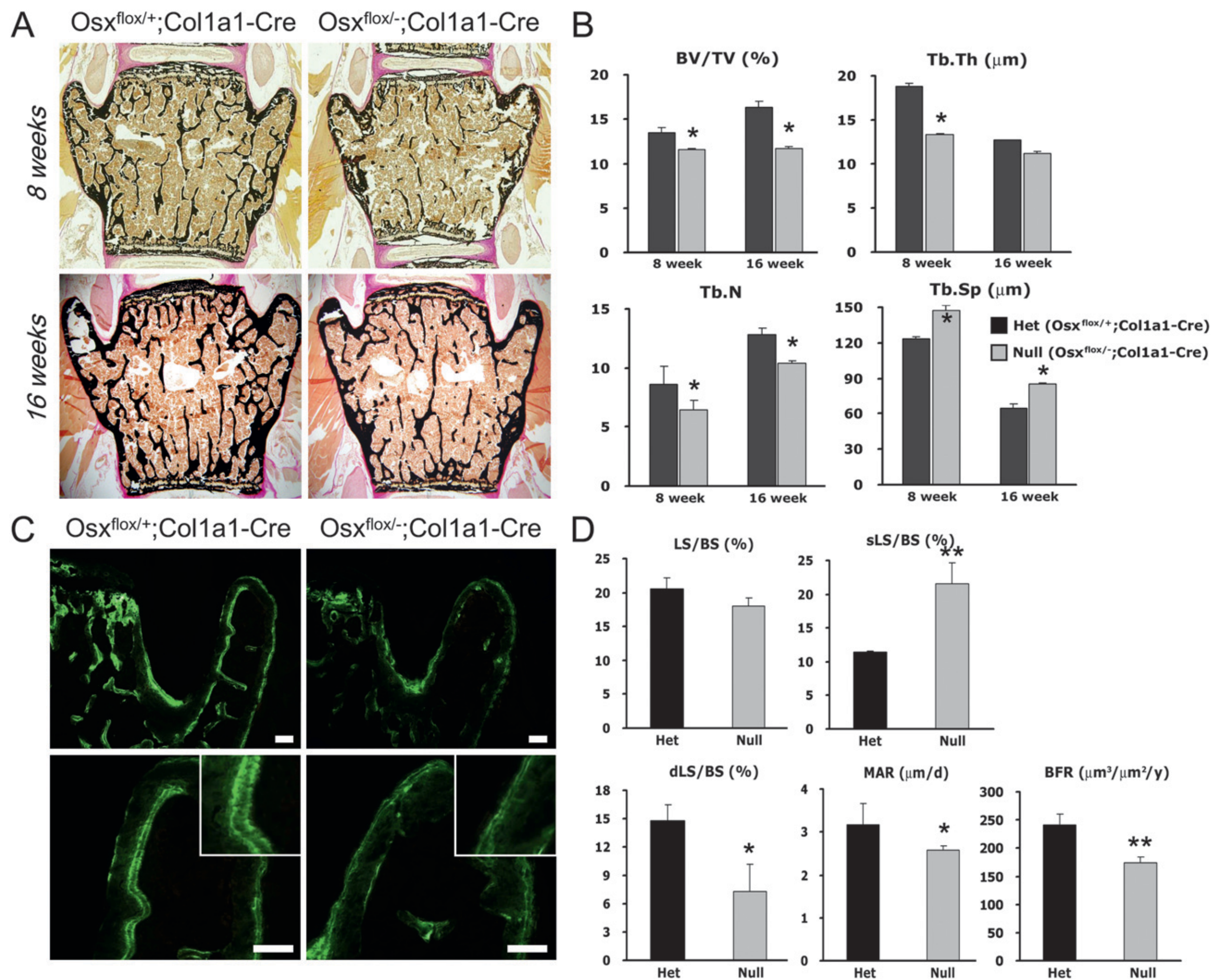

FIG. 2. Osteopenia in adult vertebra of $Osx^{flox/-}$*;Col1a1-Cre*. (A) Histological analysis of vertebrae from *Osx*-inactivated mice with *Col1a1-Cre*. The lumbar vertebrae of mice at 8 and 16 wk of age were analyzed with von Kossa staining, showing mineralized bones in black. Reduced mineralized trabecular bones were observed in $Osx^{flox/-}$*;Col1a1-Cre*. (B) Microarchitecture parameters in *Osx*-inactivated mice with *Col1a1-Cre*. Decreased cancellous bone volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N) were measured in lumbar vertebrae of $Osx^{flox/-}$*;Col1a1-Cre* mice using TAS and Bioquant program. $*p < 0.05$. (C) Reduced BFR in vertebrae of $Osx^{flox/-}$*;Col1a1-Cre*. Mice at 8 wk of age were labeled with calcein. Calcein double labeling was not obvious, and the distances between the double labeling were clearly reduced in $Osx^{flox/-}$*;Col1a1-Cre*. Images in the bottom column are the higher-magnification images corresponding to the top column. Scale bar = 100 μm. (D) Measurements based on fluorescent calcein labeling of mice. Dynamic indices of bone formation were quantitated by OsteoMeasure program. LS/BS, labeling surface; sLS/BS, single labeling surface; dLS/BS, double labeling surface. $*p < 0.05$; $**p < 0.001$.

not visibly affected (data not shown). These results showed that *Osx* inactivation in osteoblasts delayed cortical bone formation and rendered trabecular bone formation immature or premature because of osteoblast dysfunction.

No functional defect in bone resorption by osteoclasts in $Osx^{flox/-}$*;Col1a1-Cre*

In the process of normal bone ossification, matrix degradation by bone-resorbing osteoclasts and bone matrix as well as cartilage mineralization by bone-forming osteoblasts are tightly coordinated.[6] Thus, TRACP^{+} osteoclasts were comparatively studied in trabecular regions from $Osx^{flox/+}$*;Col1a1-Cre* and $Osx^{flox/-}$*;Col1a1-Cre* at 8 and 16 wk of age. TRACP^{+} osteoclasts were detected in the periosteum, cartilage matrix, and trabecular bone surfaces of $Osx^{flox/+}$*;Col1a1-Cre* and $Osx^{flox/-}$*;Col1a1-Cre*. Moreover, multinucleated cells representing functional osteoclasts were identical in both mice (Fig. 5A). The bone resorption aspect of bone remodeling was also analyzed by measuring the urinary elimination of DPD cross-links, a biochemical marker of bone resorption. No difference in osteoclast activity for bone resorption was found in $Osx^{flox/-}$*;Col1a1-Cre* compared with $Osx^{flox/+}$*;Col1a1-Cre* mice (Fig. 5B). These results indicated that *Osx* deficiency in osteoblasts did not affect osteoclast differentiation and function and that osteopenic phenotype in $Osx^{flox/-}$*;Col1a1-Cre* was not caused by bone resorption.

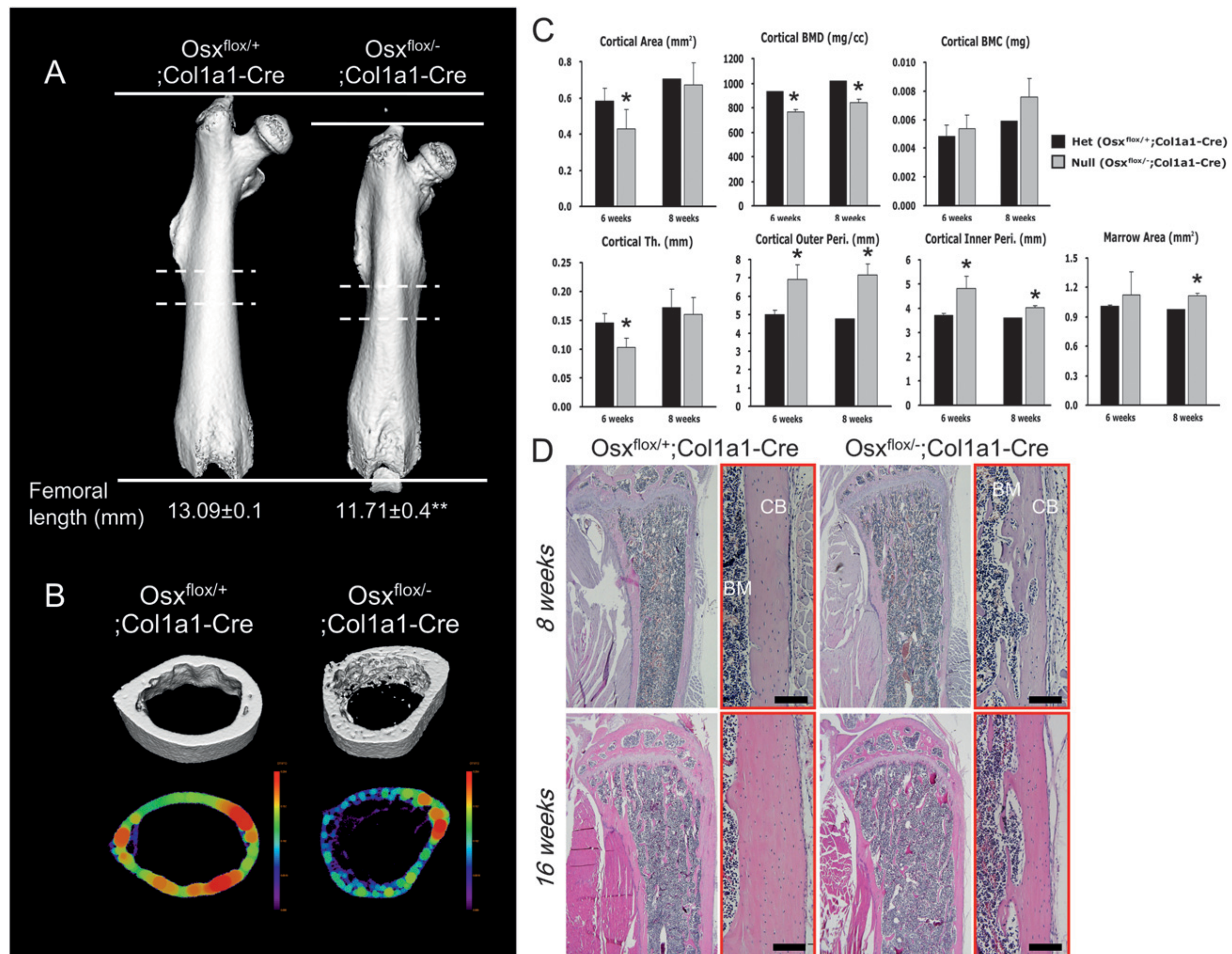

FIG. 3. Analysis of cortical bone architecture in *Osx*$^{flox/-}$*;Col1a1-Cre*. (A) Qualitative 3D μCT imaging of femoral bone at 6 wk of age. Femoral length was reduced in *Osx*$^{flox/-}$*;Col1a1-Cre* compared with *Osx*$^{flox/+}$*;Col1a1-Cre*. $^{**}p < 0.01$. (B) μCT analysis in diaphysial transverse sections of cortical bone from dashed lines in A. More porous osteopenic cortical bone was observed in *Osx*$^{flox/-}$*;Col1a1-Cre*. In analysis of femoral diaphysis by peripheral QCT, colored regions represented the cortical thickness of femoral diaphysis. The reduction in cortical bone thickness was observed in *Osx*$^{flox/-}$*;Col1a1-Cre*. (C) Histomorphometrical analysis of cortical bone at 6 and 8 wk of age. Compared with *Osx*$^{flox/+}$*;Col1a1-Cre* mice, cortical area, BMD, and thickness (Th) were decreased in *Osx*$^{flox/-}$*;Col1a1-Cre* mice, whereas cortical outer and inner peri- and marrow area were increased. $^{*}p < 0.05$. (D) Histological examination of tibias stained with H&E in *Osx*$^{flox/-}$*;Col1a1-Cre*. *Osx*$^{flox/-}$*;Col1a1-Cre* mice had thinner and more porous cortical bone resulting from the reduced bone formation. Each right panel (red box) was magnified in each left panel. BM, bone marrow; CB, cortical bone. Scale bar = 200 μm.

Hence, bone resorption is likely not the causative factor for the reduced bone formation in *Osx*$^{flox/-}$*;Col1a1-Cre*.

Altered expression of osteoblast differentiation marker genes in Osx$^{flox/-}$*;Col1a1-Cre*

Based on the in vivo results for the reduced function of osteoblasts and the lack of functional defects in osteoclasts, quantitative real-time PCR analysis was performed to identify the expression of marker genes related to osteoblast and osteoclast differentiation in long bones of *Osx*$^{flox/-}$*;Col1a1-Cre* (Fig. 6A). To verify complete *Osx* excision in *Osx*$^{flox/-}$*;Col1a1-Cre*, the expression of *Osx* gene was quantified. Compared with that in wildtype mice, *Osx* expression was at most 50% in *Osx*$^{flox/+}$*;Col1a1-Cre* heterozygous mice and <10% in *Osx*$^{flox/-}$*;Col1a1-Cre* null mice, indicating that the activity of Cre recombinase was >90% in *Osx*-inactivated mice with the *Col1a1-Cre* transgene. Osteocalcin, a marker gene at a late stage of osteoblast differentiation, was decreased by 80% in *Osx*$^{flox/-}$*;Col1a1-Cre* compared with *Osx*$^{flox/+}$*;Col1a1-Cre*. In contrast, the mRNA expression level of the pre-osteoblast marker gene, alkaline phosphatase, and of an early marker gene of osteogenic differentiation, osteopontin, in *Osx*$^{flox/-}$*;Col1a1-Cre* was significantly higher than in *Osx*$^{flox/+}$*;Col1a1-Cre*, whereas bone sialoprotein and *Col1a1* were not affected. This indicated that osteoblasts differentiation was drastically reduced and that immature osteoblasts were increased in *Osx*$^{flox/-}$*;Col1a1-Cre*. Interestingly, the mRNA expression of *Runx2*, which is upstream of *Osx*, was increased in *Osx*$^{flox/-}$*;Col1a1-Cre*. Although the exact mechanism for the high level of *Runx2* is

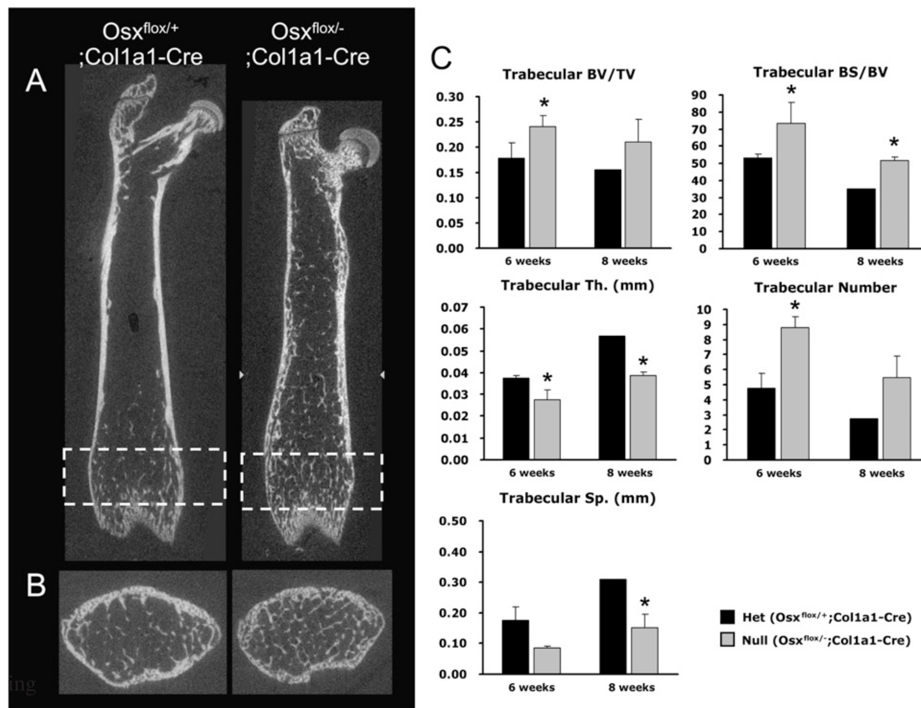

FIG. 4. Analysis of trabecular bone architecture in *Osx*$^{flox/-}$;*Col1a1-Cre*. (A) 2D longitudinal image by μCT analysis of femoral bone at 6 wk of age. Osteopenic cortical bone and small pieces of trabecular bones were observed in *Osx*$^{flox/-}$;*Col1a1-Cre*. (B) μCT analysis of rectangled metaphysis in A. Increased immature or premature trabecular bones were observed with the osteopenic cortical bone in *Osx*$^{flox/-}$;*Col1a1-Cre*. (C) Histomorphometrical analysis of trabecular bone at 6 and 8 wk of age. Compared with *Osx*$^{flox/+}$;*Col1a1-Cre* mice, trabecular bone volume (BV/TV) and bone surface (BS/BV) were increased in *Osx*$^{flox/-}$;*Col1a1-Cre* mice, accompanied by increased trabecular numbers. However, trabecular thickness (Th) and separation (Sp) were significantly reduced in *Osx*$^{flox/-}$;*Col1a1-Cre*, indicat-immature or premature trabecular bones. *$p < 0.05$.

not clear, it may be partly caused by the lack of *Osx*-mediated negative feedback mechanism or a compensation for bone formation lacking *Osx*.

To confirm the change in osteoblast differentiation, the in vitro differentiation was studied in primary calvarial osteoblasts of *Osx*$^{flox/-}$;*Col1a1-Cre*. The number of mineralized bone nodules was remarkably reduced in *Osx*$^{flox/-}$;*Col1a1-Cre* as shown by alizarin red and von Kossa staining (Fig. 6B). Because of the altered osteoblast differentiation, proliferation and apoptosis of osteoblastic cells were analyzed by BrdU incorporation and TUNEL staining in *Osx*$^{flox/-}$;*Col1a1-Cre*, respectively. Highly increased cells with BrdU incorporation were observed in *Osx*$^{flox/-}$;*Col1a1-Cre* (Fig. 6C). Even though TUNEL^{+} cells were slightly increased, no significant difference was statistically examined in *Osx*$^{flox/-}$;*Col1a1-Cre* compared with *Osx*$^{flox/+}$;*Col1a1-Cre* (Fig. 6D and data not shown). Therefore, these results indicated that proliferation of the immature osteoblasts, which accumulated because of reduced differentiation, was increased in the long bones of *Osx*$^{flox/-}$;*Col1a1-Cre*.

With the increase of immature osteoblasts, RANKL and OPG, which are important for osteoclastogenesis and osteoclast activity,[23] were also shown to be increased (Fig. 6A). The increased level of *RANKL* may reflect the relative increase in immature pre-osteoblast cells because *Osx* inactivation seemed to decelerate osteoblast differentiation and an increased *OPG* may be a compensatory mechanism to prevent bone loss in *Osx*$^{flox/-}$;*Col1a1-Cre*. No significant changes were observed in the expression of the osteoclast differentiation markers, *TRACP*, *CathK*, and *MMP9* (Fig. 6A). In conclusion, we observed osteopenia with decreased bone formation and irregular cortical bone structure in *Osx*$^{flox/-}$;*Col1a1-Cre* caused by increased immature osteoblasts and reduced osteoblast differentiation, without apparent defects in bone resorption.

DISCUSSION

The transcription factor Osx is essential for osteoblast differentiation during embryonic development, because mice lacking *Osx* show a complete absence of intramembranous and endochondral bone formation.[13] However, perinatal lethality by *Osx* gene disruption prevents studies of the role of Osx in adult bones. In this study, we provide evidence indicating that *Osx* positively regulates bone formation and maintenance after birth. To study the possible role of Osx in adult bones, we generated *Osx*-inactivated mice with *Col1a1-Cre* (*Osx*$^{flox/-}$;*Col1a1-Cre*), in which the *Osx* gene was inactivated in all osteoblasts after bone collar formation during development. *Osx*$^{flox/-}$;*Col1a1-Cre* newborn mice showed no significant abnormality compared with *Osx*$^{flox/+}$;*Col1a1-Cre* mice. However, adult mice at 8 and 16 wk of age showed an osteopenic phenotype consisting of morphological changes in trabecular and cortical bone phenotypes in long bones. Furthermore, reduced BMD and BFR were reported in the lumbar vertebrae. The cortical bone of the diaphysis of long bones was thinner and more porous in *Osx*$^{flox/-}$;*Col1a1-Cre*, developing osteopenia with a subsequent loss of bone mass. These results indicated that abnormal bone formation in adults was caused by remodeling errors.

We observed an unexpected increase in the trabecular bone phenotype of the tibia and a decrease in the length of all long bones. These phenotypes of *Osx*$^{flox/-}$;*Col1a1-Cre*

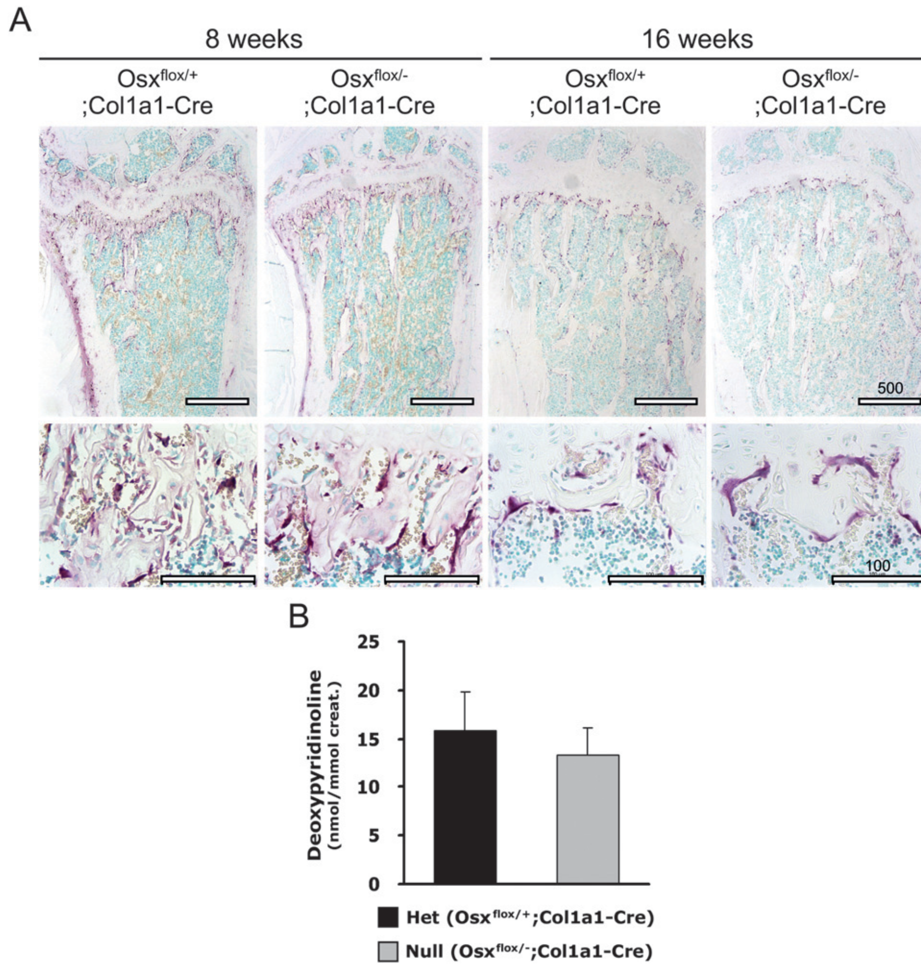

FIG. 5. No functional defect in osteoclastogenesis and bone resorption in *Osx$^{flox/-}$;Col1a1-Cre*. (A) TRACP staining was performed in tibias to detect mature osteoclasts. TRACP^{+} osteoclasts (red) were observed on the surface of bone in *Osx$^{flox/-}$;Col1a1-Cre* with no difference compared with *Osx$^{flox/+}$;Col1a1-Cre*. Scale bar = 100 μm. (B) Urinary DPD cross-links were measured as an osteoclast activity parameter in vivo. No difference in osteoclast activity for bone resorption was observed in *Osx$^{flox/-}$;Col1a1-Cre* compared with *Osx$^{flox/+}$;Col1a1-Cre* mice. The values were normalized to creatinine concentration excreted in the urine.

mice were similar to existing findings showing that abnormal trabecular and cortical bone regulatory mechanisms were responsible for longitudinal growth of the cortex as well as coalescence processes of endochondral trabecular bone.[24–26] Previous studies have reported that the trabecular bone at the periphery of the growth plate coalesces into cortical bones, whereas the central trabecular bone under the growth plate is resorbed to create the bone marrow cavity. In histomorphometrical analysis, they observed that the former was especially caused by increased bone formation by osteoblasts and not by decreased bone resorption by osteoclasts.[24,26] Thus, longitudinal growth of bone is affected to the coalescence of metaphyseal trabecular bone into cortical bone with increased osteoblast function for bone formation. This also means that the reduced bone growth results from the delayed coalescence of trabecular bone caused by decreased osteoblast function. Consistent with their reports, our results indicate that reduced longitudinal growth in *Osx$^{flox/-}$;Col1a1-Cre* mice was caused by delayed peripheral trabecular bone development by decreased osteoblast function without dysfunction of chondrogenesis. Moreover, even though there was no significant difference in the cortical bone formation in newborn mice, slight differences at postnatal day 3 (P3) and obvious differences at P7 were observed between *Osx$^{flox/+}$;Col1a1-Cre* and *Osx$^{flox/-}$;Col1a1-Cre* mice (data not shown). This was also confirmed by histological and μCT analysis; trabecular and cortical bones of young *Osx$^{flox/+}$;Col1a1-Cre* and adult *Osx$^{flox/-}$;Col1a1-Cre* showed a similar phenotype (data not shown). The early-stage and immature osteoblasts were highly increased in adult *Osx$^{flox/-}$;Col1a1-Cre*, as indicated by the accumulation of osteopontin-positive cells and a decrease of osteocalcin expression in the analysis of quantitative real-time PCR. This observation suggested that delayed trabecular bone and osteopenic cortical bone formation resulted from the inhibition of osteoblast differentiation and the accumulation of immature osteoblasts in adult mice without *Osx*.

Runx2 is a well-characterized bone transcription factor that is requisite for the maturation of hypertrophic chondrocytes and osteoblasts, whereas *Osx* is a downstream gene of *Runx2*.[9,10,27,28] Interestingly, in *Osx*-inactivated adult mice, *Runx2* expression was significantly increased. Although the exact mechanism is not yet clear at this moment, increased *Runx2* expression may be partly caused by the lack of *Osx*-mediated negative feedback mechanism and its expression may be controlled by the activation of the *Osx*-mediated gene. Affymetrix Genechip analysis showed increases of bone factors including *Runx2* in *Osx$^{flox/-}$;Col1a1-Cre* (unpublished data). Various factors

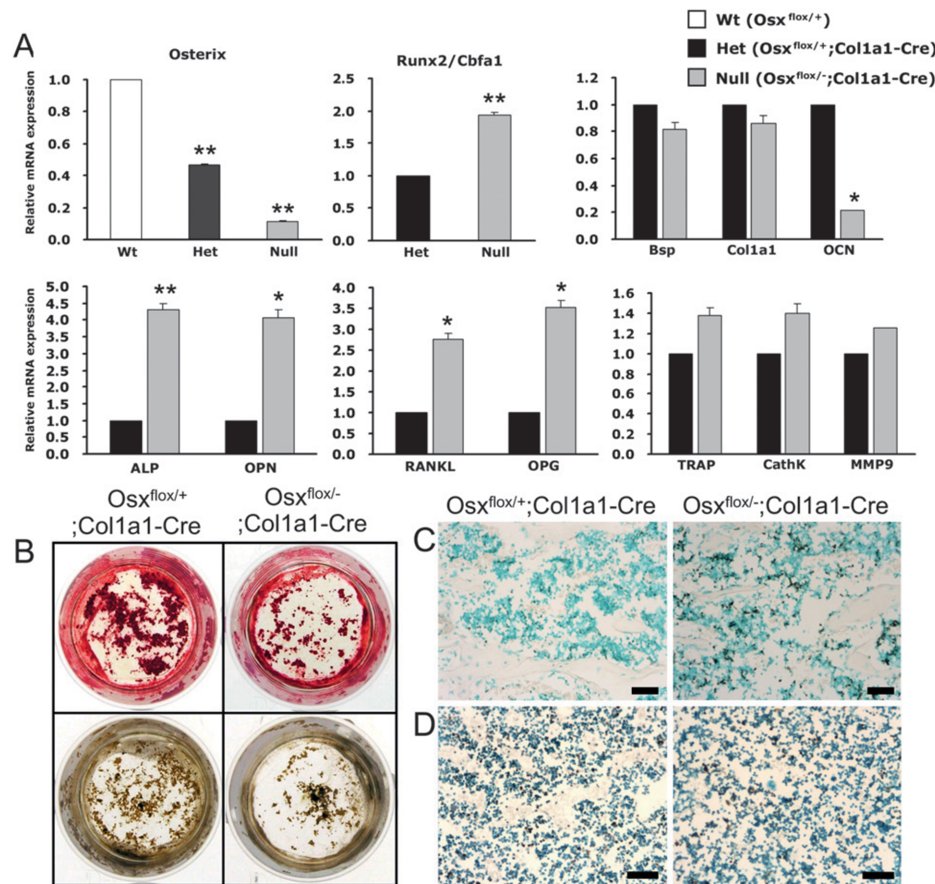

FIG. 6. Bone cell differentiation in *Osx*$^{flox/–}$*;Col1a1-Cre*. (A) Expression of marker genes related to bone cell differentiation by quantitative real-time PCR analysis. The expression of *Osx* was decreased by up to 90% in *Osx*$^{flox/–}$*;Col1a1-Cre* compared with *Osx*$^{flox/+}$ mice. In *Osx*$^{flox/–}$*;Col1a1-Cre* mice, the expression of *OCN*, a marker gene at a late stage of osteoblast differentiation, was obviously reduced, whereas the expression of the preosteoblast marker genes, *ALP* and *OPN*, was significantly increased. The expression of *OPG* and *RANKL* was high, and the expression of osteoclast differentiation markers including *TRACP*, *CathK*, and *MMP9* was not significantly changed. $^{*}p < 0.05$; $^{**}p < 0.001$. (B) Reduced differentiation in primary calvarial osteoblasts. Differentiated osteoblasts were justified by alizarin red (top) and von Kossa (bottom) staining. The number of mineralized bone nodules was remarkably reduced in *Osx*$^{flox/–}$*;Col1a1-Cre*. (C and D) Increased proliferating cells and unaltered apoptotic cells were observed in *Osx*$^{flox/–}$*;Col1a1-Cre* at 8 wk of age with BrdU and TUNEL staining (positive cells in black), respectively. Scale bar = 50 μm.

for bone formation were significantly increased to compensate for the loss of *Osx* in adult bone formation, whereas matrix proteins for cartilage formation were remarkably decreased. This may also explain the increased immature or premature trabecular bones in long bones of *Osx*$^{flox/–}$*;Col1a1-Cre* mice. Furthermore, *Osx* inactivation affected osteogenic cell proliferation. A recent report also showed that BrdU^{+} cells were increased in calvaria of conventional *Osx*-null embryos, indicating that Osx regulates osteoblast proliferation.[29] A significant increase of proliferating osteoblastic cells in *Osx*$^{flox/–}$*;Col1a1-Cre* was indeed considered to be caused by impaired osteoblast maturation and accumulated immature osteoblasts.

In the tightly coupled processes of osteoblastogenesis and osteoclastogenesis, failure of osteoblast differentiation affects osteoclast maturation and function.[4,6] Whereas delayed osteoblast differentiation was observed, there was no abnormality of osteoclast differentiation and activity in *Osx*$^{flox/–}$*;Col1a1-Cre*. It has been shown that bone transcription factors are involved in the regulation of *RANKL* and *OPG* expression, which stimulates and inhibits osteoclast differentiation and activity, respectively.[23,30–32] In our study, the expression levels of both *RANKL* and *OPG* mRNA were considerably increased in *Osx*$^{flox/–}$*;Col1a1-Cre* mice. The relative ratio of *RANKL*/*OPG* was not significantly changed, and hence, there was no abnormality of bone resorption in *Osx*$^{flox/–}$*;Col1a1-Cre* mice. Eventually, increased *OPG* may compensate for osteoclast maturation and bone resorption by increased *RANKL* from accumulated immature osteoblasts, after restoration of bone homeostasis. Also, the mRNA expression of osteoclast marker genes including *TRACP*, *CathK*, and *MMP9* was unaltered, confirming that defects of endochondral ossifications in *Osx*$^{flox/–}$*;Col1a1-Cre* were not caused by the abnormality of osteoclasts function. Therefore, as depicted in Fig. 7, we concluded that, in the *Osx*-deficient bone of adults, differentiation of pre-osteoblasts into mature osteoblasts was delayed with subsequent

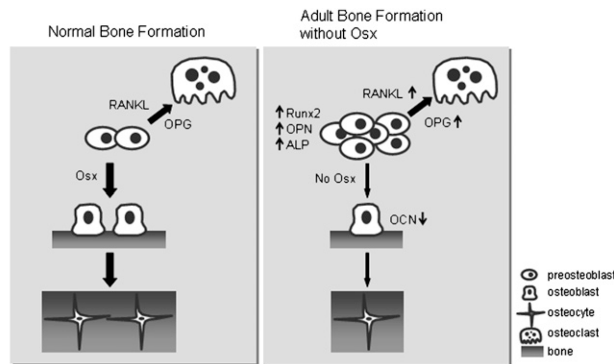

FIG. 7. Proposed model of bone formation governed by Osx in adult bone. With the lack of Osx during the animal's growth, differentiation of pre-osteoblasts into mature osteoblasts is delayed and immature osteoblasts accumulate, accompanied by the increased expression of early marker genes for osteoblast differentiation. Although bones are formed, BFR and bone mass are significantly reduced. However, *Osx*-inactivated adult mice have no obvious defect in osteoclast differentiation because the relative ratio of *RANKL*/*OPG* is not remarkably changed, even though the expression of *RANKL* and *OPG* is highly increased. Thus, Osx is a positive regulator in adult bone formation.

accumulation of immature osteoblasts. In addition, to compensate for the lack of *Osx* in bone formation, *Osx* inactivation in adult mice resulted in accelerated osteogenic cell proliferation and subsequently in premature trabecular bone formation in long bones. Even though the expression of *RANKL* and *OPG* was increased, no functional defects were observed in osteoclast differentiation because there was no change in the relative ratio of *RANKL*/*OPG*.

In summary, this study suggests that Osx is required for the regulation of osteoblast differentiation in vivo and for bone formation and maintenance in growing or already formed bones. Osx also has a key role in longitudinal bone growth by osteoblasts. Understanding the regulatory function of Osx in adult bone may shed light on the complex mechanisms of bone diseases and help address appropriate therapies for bone diseases such as osteoporosis and osteogenesis imperfecta.

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